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THE LOW-TEMPERATURE SPECTRAL PROPERTIES OF MAMMALIAN CYTOCHROME OXIDASE

I. THE ENZYME IN INTACT RAT-LIVER MITOCHONDRIA

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SUMMARY

The liquid-N_2 temperature spectra of cytochrome oxidase (cytochrome c:O_2 oxidoreductase, EC 1.9.3.1) in rat-liver mitochondria are presented. Sulfide is postulated to inhibit respiration by forming an oxidized cytochrome a_3–sulfide compound. The azide-inhibited cytochrome oxidase is characterized by the presence of a form of reduced cytochrome a which has a spectrum different from the usual spectrum of reduced cytochrome a both in the alpha and Soret regions.

No evidence was found for interaction of cytochromes a and a_3 in such a manner that either the spectrum of the reduced cytochrome a or its reactivity toward azide were affected by the valence state of cytochrome a_3.

INTRODUCTION

Cytochrome oxidase (cytochrome c:O_2 oxidoreductase, EC 1.9.3.1) has been the subject of extensive investigation for many years with respect to both its spectral and its enzymatic properties. In 1939, KEILIN AND HARTREE[1] concluded that two cytochromes of the a type (a and a_3) were present in the respiratory chain of yeast and heart muscle and that these two cytochromes had very different properties. Cytochrome a was defined as the cytochrome which did not combine with CN^-, CO or O_2 and cytochrome a_3 was defined as the cytochrome which did combine with these ligands and was identical to the "Atmungsferment" of WARBURG AND NEGELEIN[2,3]. They further concluded that the absorption band at 605 mμ was principally due to cytochrome a while the absorption band at 445 mμ was due to both cytochromes with cytochrome a_3 dominating. Since that time the absorption spectra of cytochrome oxidase have been measured by many workers[4–14], but the interpretation of the spectra remains a matter of controversy.

Disagreement exists on such fundamental concepts as whether cytochromes a

Abbreviations: TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; MSET-P_i, mannitol–sucrose–EDTA–Tris–phosphate medium.

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and a_3 represent separate cytochromes[1,4–6,11–17] each containing heme *a* but with the heme *a* groups having very different reactivities toward added ligands, or if they represent a single cytochrome[8,10,18,19] having an anomalous spectral behavior due in part to the reactive formyl group conjugated with the porphyrin nucleus of heme *a*.

The development of a technique for the trapping of the aerobic steady state of mitochondria which incorporates the increased spectral resolution of liquid-N_2 temperatures[20–23] has made available a powerful tool for studying the spectral properties of cytochromes. We have previously reported a new spectral form of cytochrome *a*[24–26] using this technique. This form of cytochrome *a* was observed when mitochondria respiring in the active state (State 3) were inhibited by azide. Its spectral properties and enzymatic behavior suggested that it would arise either as an azide compound of reduced cytochrome *a* or as an intermediate in the energy conservation reactions of mitochondria or both. The possibility of the formation of an azide compound of cytochrome *a* is inconsistent with the currently accepted idea that the iron atom of the heme group in the native cytochrome *a* is unable to undergo ligand replacement reactions and would thus require a re-evaluation of our current concepts of electron transport reactions in mitchondria. In all cases in which the cytochromes other than a_3 have been observed to undergo ligand replacement reactions, these reactions have been observed to occur only after the loss of enzymatic activity[27]. The possibility that this new form of cytochrome *a* is involved in energy transfer reactions is equally of interest as it offers an important tool in the study of the mechanism of the coupling of electron transport reactions to energy conservation reactions.

A study of the spectral properties of cytochrome oxidase in intact mitochondria by the trapped steady-state technique was undertaken in order to complement the previous work and expand our knowledge of the spectral properties of cytochrome oxidase. In the present communication, particular attention is devoted to the azide-inhibited system.

MATERIALS AND METHODS

Reagents

Sodium azide (Matheson, Coleman and Bell) or potassium azide (Eastman Organic Chemicals), potassium cyanide (J. T. Baker, reagent grade), sodium sulfide (Fisher Scientific, reagent grade), mannitol (J. T. Baker, reagent grade), sucrose (J. T. Baker, reagent grade), EDTA (Sigma Chemical Co.), Tris (Sigma Chemical Co., reagent grade), L-ascorbic acid (J. T. Baker, reagent grade), *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (Eastman Kodak Co.), and antimycin A (Sigma Chemical Co.) were used without further purification. The ascorbic acid was neutralized to pH 6.8 with NaOH prior to use.

Media

The rat-liver mitochondria were isolated in a medium containing 0.22 M mannitol, 0.075 M sucrose and 0.2 mM EDTA and a final pH of 7.2. All spectra were measured in a medium containing 0.22 M mannitol, 0.075 M sucrose, 0.2 mM EDTA, 15 mM Tris and 10 mM KH_2PO_4. This medium is abbreviated as MSET-P_i and the pH's used for the individual experiments are given in the figure and table legends.

Methods

Protein determinations were made by the biuret method[29]. Rat-liver mitochondria were prepared and the spectra were measured as previously described[28,30]. The spectrophotometer sample temperature was measured using an iron–constantan thermocouple[21,30].

RESULTS

In order to study the spectral properties of cytochrome oxidase in intact mitochondria it is necessary to eliminate, in so far as possible, spectral contributions due to other absorbing materials present in the mitchondrion. The absorption of mitochondrial components which do not change their spectral properties during mitochondrial function can be eliminated by measuring the spectral differences between two samples instead of the absolute spectra of the individual samples. Components which do change their spectral properties present a more difficult problem.

The respiratory chain of rat-liver mitochondria contains cytochromes *b*, *c* and c_1 in addition to cytochrome oxidase and each of these cytochromes can undergo absorbance changes which must be differentiated from those of cytochrome oxidase.

The observational difficulties may be seen in Fig. 1A which is a difference spectrum for a CN^--treated mitochondrial suspension *minus* a mitochondrial suspension treated to oxidize the respiratory chain carriers. Although the α band of cytochrome oxidase at 602 mμ is virtually free of spectral interference the Soret region is made up of several overlapping absorption bands. Cytochrome *b* which has a Soret band at 430 mμ contributes to the reduced cytochrome oxidase band at 440 mμ and, together with cytochromes *c* and c_1, obscures the Soret band of oxidized cytochrome oxidase which should appear as a minimum at 418 mμ. In Figs. 1B and 1C, the cytochrome *b* contribution has been minimized by two different techniques.

The electron donor system of ascorbate–TMPD donates electrons to cytochrome *c* and cytochrome *b* is reduced only in the presence of an energy source, such as the high energy intermediates formed during electron transport or in the presence of ATP[31]. Cytochrome *b* may be maintained in the oxidized form by inhibiting the oxidation of endogenous substrates and adding uncouplers of oxidative phosphorylation (Fig. 1B). The alternative technique (Fig. 1C) is to maintain the cytochrome *b* in the fully reduced form by adding antimycin A and succinate to both samples. The very small difference between the two spectra in the absorption at 586 mμ is due to slight differences in the concentrations of the reduced cytochrome a_3–CN^- compound. This is not reproducible and cannot be attributed to the presence or absence of antimycin A.

Cytochromes *c* and c_1 cannot be prevented from undergoing oxidation and reduction. When such changes occur, however, the only spectral information lost is that related to the Soret band of oxidized cytochrome oxidase. Both of the techniques used to minimize the cytochrome *b* redox changes also minimize redox changes in the mitochondrial flavoproteins. The flavoprotein reduced *minus* oxidized difference spectrum which is characterized by the broad trough centered at 465 mμ is thus also minimized (Fig. 1).

The aerobic steady state of cytochrome oxidase in the presence of its inhibitors

The effect of inhibitors of cytochrome oxidase on the spectra of the cytochromes

for mitochondrial suspensions in the aerobic steady state was determined by measuring the difference between the absorption spectra of mitochondrial suspensions in the aerobic steady state in the presence of CN^-, S^{2-}, CO or N_3^- and that of mitochondrial suspensions treated to oxidize the cytochrome oxidase (Fig. 2, A–E). The difference spectrum of an anaerobic mitochondrial suspension and that of a mitochondrial suspension treated to oxidize the cytochrome oxidase (Fig. 2A) is included for comparison with the inhibited systems. CN^- inhibition (Fig. 2B) gives a difference spectrum which differs from that for the uninhibited system in that a small peak is observed at 587 mμ and the Soret absorption has decreased by approximately one-half. Instead of

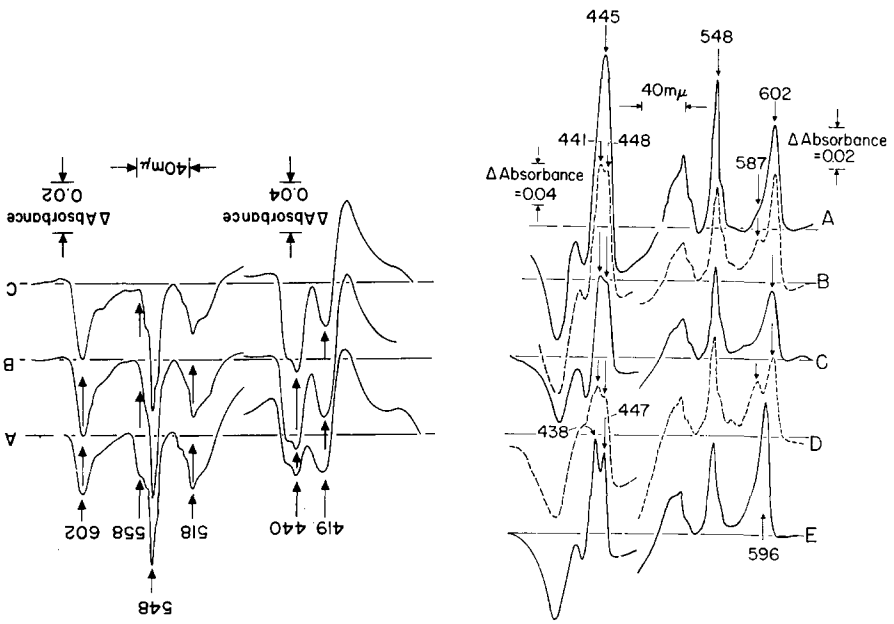

Fig. 1. The elimination of the spectral contributions resulting from redox changes of cytochrome *b* and flavoproteins. Rat-liver mitochondria were suspended in MSET-P_i (pH 7.2) at 7.0 mg protein per ml and treated as follows: Spectrum A: 20 μM dicumarol was added and the suspension aerated for 5 min at 24° and then the reference sample was frozen. The remaining suspension was made 10 mM in succinate and 1 mM in CN^-, aerated for 2 min and then the measure sample was frozen. Spectrum B: 50 μM dicumarol and 10 mM ascorbate were added, the suspension aerated for 2 min and then the reference sample was frozen. 100 μM TMPD and 1 mM CN^- were added to the remaining suspension and the measure sample was frozen after 2 min aeration. Spectrum C: 1.8 μg antimycin A per ml, 5 mM succinate and 10 mM ascorbate were added and the reference sample frozen after 1 min aeration; 100 μM TMPD and 1 mM CN^- were added to the remaining suspension and the measure sample was frozen after 2 min aeration.

Fig. 2. The difference spectra for cytochrome oxidase in the aerobic steady state in the presence of its inhibitors *minus* oxidized cytochrome oxidase. The rat-liver mitochondria were suspended in MSET-P_i medium (pH 7.2) at 5.5 mg protein per ml. 1.8 μg antimycin A per ml, 10 mM succinate and 15 mM ascorbate were added, the suspension was aerated for 2 min and then the reference samples were frozen. Further additions were made for the measure samples as follows: Spectrum A: 400 μM TMPD was added and the suspension sealed in a syringe. The sample was frozen after anaerobiosis. Spectrum B: 400 μM TMPD and 1 mM CN^- were added and the suspension aerated for 2 min, then the sample was frozen. Spectrum C: 400 μM TMPD and 2 mM S^{2-} was added and the suspension was aerated for 1 min, then the sample was frozen. Spectrum D: 400 μM TMPD was added and CO gas bubbled through the suspension for 20 sec and then the sample was frozen. Spectrum E: 400 μM TMPD and 1 mM N_3^- were added and the suspension aerated for 1 min, then the sample was frozen.

a single smooth maximum at 443 mμ, the Soret band has two poorly separated maxima at 441 and 448 mμ. The small maximum at 587 mμ may be attributed to the presence of a small amount of reduced cytochrome a_3–CN^- compound[1,12]. The changes in the Soret maximum indicate that most of the cytochrome a_3 is oxidized in the aerobic steady state, probably existing as the oxidized cytochrome a_3–CN^- compound[1,12]. The observed absorption maxima are due to the split Soret band of cytochrome *a* which has been previously described.

S^{2-} has been increasingly used as a terminal inhibitor[23], but its site of inhibition has not been studied extensively since the work of KEILIN AND HARTREE[1]. As may be seen from Fig. 2C, the aerobic steady state of the S^{2-}-inhibited system is very similar to that of the CN^--inhibited system, except that there is no evidence for the presence of a reduced cytochrome a_3–S^{2-} compound. The cytochrome *a* absorption spectrum is the same for S^{2-} and CN^- inhibition, suggesting that S^{2-}, like CN^-, inhibits by forming an inhibitory compound of oxidized cytochrome a_3 as suggested by KEILIN AND HARTREE[1].

CO is known to inhibit respiration by competing with O_2 for a binding site on reduced cytochrome a_3 (refs. 1, 32). As may be seen from Fig. 2D, in the aerobic steady state the cytochrome *a* spectrum is the same as that in the cyanide and sulfide inhibited systems. The reduced cytochrome a_3–CO compound has absorption maxima at 587 mμ and 428 mμ (ref. 33), the latter appearing as a shoulder on the 441-mμ absorption maximum of cytochrome *a*.

The difference spectrum for the aerobic steady state of the N_3^--inhibited system (Fig. 2E) differs strikingly from those for CN^-, S^{2-} and CO. The cytochrome a_3 is apparently in the oxidized form as it was for CN^- and S^{2-}, but the spectrum of cytochrome *a* has been markedly altered. The alpha absorption maximum is at 596 mμ instead of 602 mμ and the Soret absorption band has two well-defined maxima at 438 mμ and 447 mμ instead of two poorly separated maxima at 441 mμ and 448 mμ.

The difference spectra for anaerobic mitochondrial suspensions minus mitochondrial suspensions in the aerobic steady state in the presence of terminal inhibitors

Each of the inhibitors was observed to cause a marked loss of absorption in the Soret region of the spectrum (Fig. 2) with respect to the spectrum of the anaerobic mitochondrial suspension. A comparison of the absorption spectra of the aerobic steady states of the terminally inhibited mitochondria may be made by directly measuring the difference spectrum for anaerobic mitochondrial suspensions in the absence of the inhibitor *minus* mitochondrial suspensions in the aerobic steady state (Fig. 3).

CN^- and S^{2-} give very similar difference spectra (Fig. 3A,B) with a small maximum between 606 and 609 mμ and a strong maximum at 444–445 mμ. The broad trough in the difference spectra centered at 465 mμ is not a property of cytochrome oxidase but appears to result from a more complete reduction of the mitochondrial flavoproteins in the anaerobic suspension. There are some differences between the spectra but these are small and may represent spectral differences between the respective compounds with oxidized cytochrome a_3 which are present in the aerobic steady state.

CO behaves in the expected manner and the difference spectrum (Fig. 3C) has a maximum at 446 mμ and minima at 587 mμ and 426 mμ, representing the difference

spectrum for reduced cytochrome a_3 *minus* the reduced cytochrome a_3–CO compound.

The difference spectrum for the anaerobic mitochondrial suspension *minus* mitochondrial suspension in the aerobic steady state in the presence of N_3^- is markedly different from those for S^{2-}, CN^- and CO. The alpha band of cytochrome *a* is at a longer

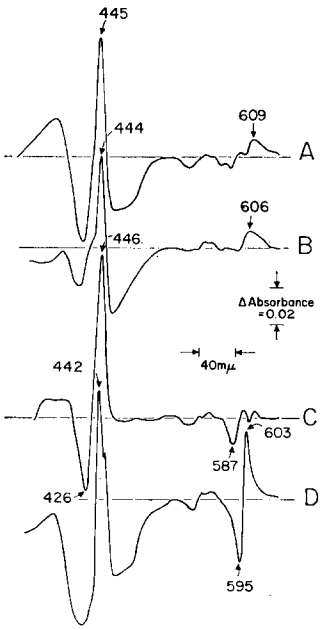

Fig. 3. The difference spectra of fully reduced cytochrome oxidase *minus* cytochrome oxidase in the aerobic steady state in the presence of its inhibitors. The rat-liver mitochondria were suspended in MSET-P_i medium (pH 7.2) at 5.5 mg protein per ml. 1.8 μg antimycin A per ml, 10 mM succinate, 15 mM ascorbate and 400 μM TMPD were added and the measure samples were frozen after anaerobiosis. The reference samples were treated as follows: Spectrum A: 1 mM CN^- was added and the suspension was aerated for 2 min and then it was frozen. Spectrum B: 2 mM S^{2-} was added and the suspension was aerated for 1 min and then it was frozen. Spectrum C: CO gas was bubbled through the suspension for 30 sec and then the sample was frozen. Spectrum D: 1 mM N_3^- was added and the suspension aerated for 1 min and then the sample was frozen.

wavelength in the fully reduced sample than it is in the aerobic state as indicated by the maximum at 603 mμ and the minimum at 595 mμ. In the Soret region, a strong maximum is observed at 442 mμ with a secondary maximum at 448 mμ. This apparent asymmetry of the reduced cytochrome a_3 maximum may be attributed to the difference between the reduced cytochrome *a* spectrum in the N_3^--inhibited aerobic steady state and "normal" reduced cytochrome *a* (see Fig. 2).

The requirement for electron flux for the azide-induced change in the spectrum of cytochrome a

The spectrum of cytochrome *a* in an N_3^--inhibited mitochondrial suspension has been shown[24–26,28] to be the form of cytochrome having an alpha band at 596 mμ in the reduced *minus* oxidized difference spectrum (cytochrome a_{596}) even when the mitochondria were respiring in the presence of uncouplers or 2 % cholate. The change from cytochrome a_{596} to cytochrome a_{602} occurred only after anaerobiosis. It was

further observed that for tightly coupled mitochondria in State 3 in the presence of N_3^-, the reduction of cytochrome a_3 was very slow after anaerobiosis, but its rate of reduction could be greatly increased by adding uncouplers of oxidative phosphorylation.

The cytochrome a_{596} to cytochrome a_{602} transition was observed to coincide with the reduction of cytochrome a_3. This observation suggested the hypothesis that the valence state of cytochrome a_3 determined the ability of N_3^- to induce the formation of cytochrome a_{596}. The terminal inhibitors CN^-, S^{2-} and CO may be used to test such a hypothesis because the first two bind the oxidized form of cytochrome a_3 while the last binds the reduced form of cytochrome a_3. The difference spectra of the aerobic mitochondrial suspensions were measured in which the reference sample contained mitochondria in the N_3^--inhibited steady state and the measured sample contained mitochondria in the N_3^--inhibited steady state to which CN^-, S^{2-} or CO was also added. As may be seen in Fig. 4, the difference spectra for the three terminal inhibitors

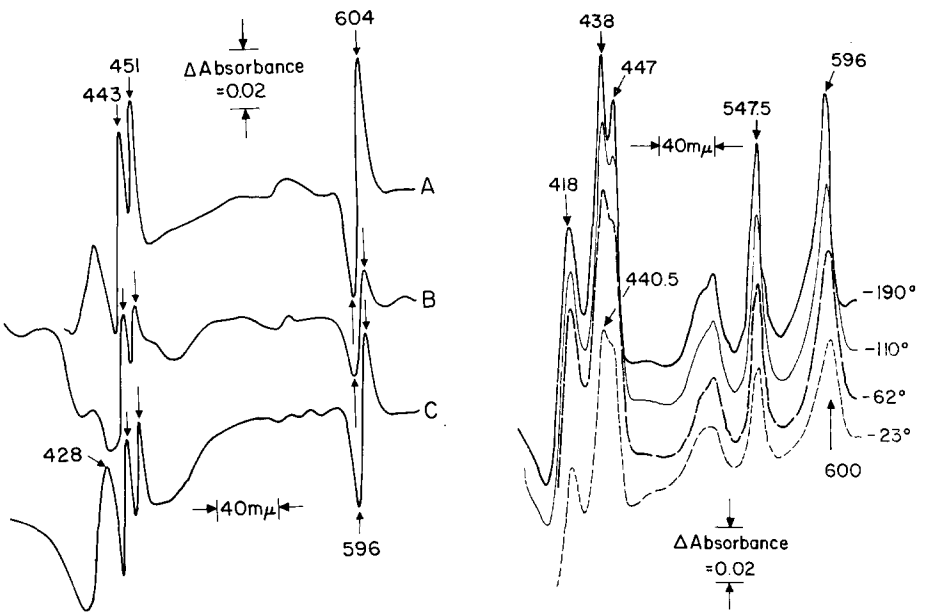

Fig. 4. The reversal of the N_3^--induced spectral shift in cytochrome *a* by inhibitors acting between the site of N_3^- inhibition and O_2. The rat-liver mitochondria were suspended in MSET-P_i medium (pH 7.2) at 5.5 mg protein per ml. 1.8 μg antimycin A per ml, 10 mM succinate, 15 mM ascorbate, 400 μM TMPD and 500 μM N_3^- were added and the suspension aerated for 1 min and then the reference samples were frozen. The reference samples were the same for all the spectra in this figure. The measure samples were prepared by treating the N_3^--inhibited suspension as follows: Spectrum A: 1 mM CN^- was added and the suspension aerated for 2 min and then it was frozen. Spectrum B: 2 mM S^{2-} was added and the suspension aerated for 2 min, then it was frozen. Spectrum C: CO gas was bubbled through the suspension for 30 sec and it was sealed in a syringe for 3 min to exclude O_2 diffusion and then it was frozen.

Fig. 5. The temperature dependence of spectrum of cytochrome oxidase. The rat-liver mitochondria were suspended in MSET-P_i medium (pH 7.3) at 15 mg protein per ml and 0.59 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB) added. After aeration for 5 min the reference sample was frozen, 20 mM succinate and 1 mM N_3^- were then added to the remaining suspension and the measure sample frozen after 30 min aeration. The sample temperature was continuously monitored with an copper–constantan thermocouple and the temperature given in the figure is the mean temperature during the recording of the spectrum.

have marked similarities. They show a sharp maximum at 604 mμ and a minimum at 596 mμ in the alpha region and two small sharp maxima at 443 and 451 mμ in the Soret region. The three inhibitors did not cause the same degree of cytochrome a_{596} to cytochrome a_{602} transition as seen by the spectra presented in Fig. 4, but this is a function of the experimental conditions and is an expression of the relative inhibitory power of the various reagents under the experimental conditions used. That is, a greater spectral change was observed for S^{2-} when either less N_3^- or more S^{2-} was used.

The temperature dependence of the spectrum of cytochrome oxidase

The split Soret band of cytochrome *a* is most unusual and the splitting is sufficiently large for cytochrome a_{596} (9 mμ) that it is surprising that it has not been observed in room temperature spectra. A partial explanation for this is seen in Fig. 5 in which the reduced *minus* oxidized difference spectrum of the N_3^--inhibited cytochrome oxidase is shown as a function of temperature. As the temperature was increased the 438-mμ absorption maximum shifted to longer wavelengths while the 447-mμ maximum did not change position. At room temperature the 438-mμ band had shifted to 442-mμ and the 447 mμ maximum appeared as a slight shoulder on the long wavelength side of the absorption band.

DISCUSSION

Liquid-N_2-temperature spectra of cytochrome oxidase *in situ* show marked differences between the reduced *minus* oxidized difference spectra of cytochrome *a* and a_3 (refs. 24–26). Cytochrome *a* is characterized by having a Soret absorption band which is split and appears as two maxima and a strong alpha band at 602 mμ while cytochrome a_3 has a single Soret absorption maximum and a weak alpha band. The two cytochromes are very closely related functionally and conformational changes, as measured by optical rotatory dispersion, occur during oxidation and reduction of isolated cytochrome oxidase[34].

Conformational changes in the protein structure may give rise to measurable changes in the cytochrome absorption spectrum and, therefore, it was of interest to see if the redox state of cytochrome a_3 influenced the absorption spectrum of reduced cytochrome *a*. In the present work no change in the cytochrome *a* spectrum was detected between the aerobic steady states with S^{2-} and CN^- in which the cytochrome a_3 was oxidized and the CO-inhibited state in which the cytochrome a_3 was reduced. If a physical interaction exists between the heme groups of cytochromes *a* and a_3 it is not expressed in the absorption spectrum of reduced cytochrome *a*.

It has been previously observed[26,28] that when uncouplers of oxidative phosphorylation were added to anaerobic N_3^--inhibited mitochondria, the N_3^--induced spectral shift was released with a concomitant reduction of cytochrome a_3. One possible interpretation of this observation was that the reactivity of cytochrome *a* to N_3^- was dependent on the valence state of cytochrome a_3. That is, that N_3^- could react with reduced cytochrome *a* when cytochrome a_3 was in the oxidized form but when the cytochrome a_3 was reduced a structural change occurred in the region of the cytochrome *a* heme group which altered its ability to react with N_3^-. This interpretation has now been conclusively eliminated by the observation that the N_3^--induced spectral change in reduced cytochrome *a* is reversed by inhibitors reacting between

the N_3^- site and O_2. The reversal occurs whether the cytochrome a_3 is in the oxidized (CN^- and S^{2-}) or reduced (CO and anaerobiosis) form.

The Soret band of the cytochrome *a* of rat-liver mitochondria as observed in the terminally inhibited mitochondria (Fig. 1, B–D) has maxima at 441 mμ and 448 mμ. The spectrum of cytochrome *a* of sea-urchin sperm, which was first used to demonstrate the splitting of the Soret band in the absence of added inhibitors of cytochrome oxidase[24,25], has maxima at 438 mμ and 447 mμ It therefore differs slightly quantitatively but not qualitatively from the spectrum of the cytochrome *a* of rat liver under the experimental conditions used.

A comparison of the spectral properties of cytochrome oxidase inhibited with N_3^- to the spectral properties of the CN^--, S^{2-}- and CO-inhibited enzyme show that N_3^- alone modifies the spectrum of cytochrome *a*. The presence of N_3^- modifies the spectrum of cytochrome *a* in both the alpha and Soret regions. The alpha band is shifted from 602 mμ to 596 mμ and the two Soret maxima are at 438 mμ and 447 mμ in the N_3^--inhibited form instead of 441 mμ and 448 mμ as in the "normal" reduced form. Reduced cytochrome *a* must be able to exist in two forms differing in chemical reactivity, one form permitting an N_3^--induced spectral change and the other form insensitive to N_3^- (ref. 26). The actual reaction site of N_3^- need not be the heme iron but can be some component of the energy-transfer reactions. The magnitude of the N_3^--induced spectral shift is, however, similar to the spectral changes observed by LEMBERG and co-workers[35,36] for some ligand changes in heme *a* compounds.

Recently it has been reported by MUIJERS, VAN GELDER AND SLATER[37] that the N_3^- reacts with isolated cytochrome oxidase to give an oxidized cytochrome a_3–N_3^- compound. The significance of this observation is unclear since the oxidized cytochrome a_3–N_3^- compound required 10^4 times as long to form as is required for the inhibitory compound[38] (19 h *vs.* a few seconds) and the calculated binding constant is 10^2 as large as the apparent inhibitor constant[38] (6.3 mM *vs.* 53 μM). In addition, the N_3^- concentration dependence (*n*) was 0.5 for the formation of the oxidized cytochrome *a*–N_3^- compound, but appears to be 1 for the inhibition of respiration[26]. These differences are so large that it must be tentatively concluded that the observed oxidized cytochrome a_3–N_3^- compound is not related to the inhibition of respiration by N_3^-.

It is reasonable to propose that during active respiration cytochrome *a* cycles through a labile reduced form which is capable of undergoing ligand replacement reactions or at least modification reactions. This labile reduced form of cytochrome *a* may be an integral part of both the electron transport and energy conservation reactions of mitochondrial oxidations. Uncoupling of oxidative phosphorylation at the terminal site may be directly related to the ability of the uncouplers to catalyze the destruction of this labile form of cytochrome *a* (ref. 26).

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